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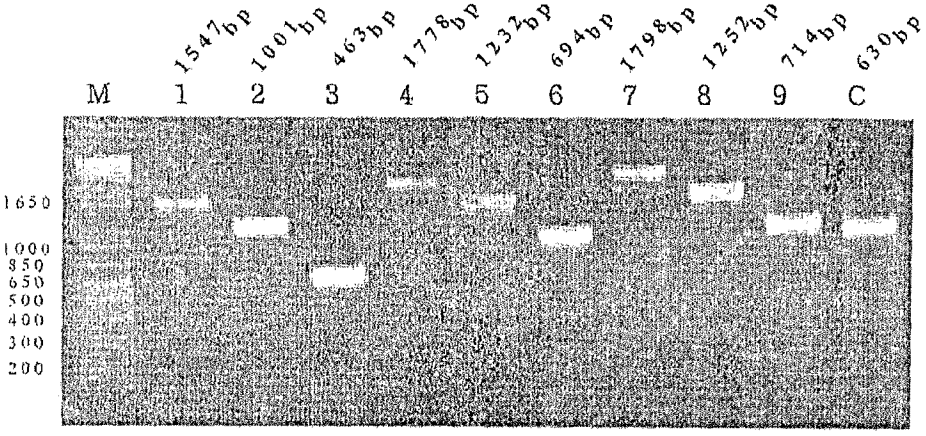


(57) Abstract: Disclosed is a screening method of genes of reassortant influenza viruses in a reassortant influenza virus preparation process by using a difference between primers in binding specificity to templates in multiplex RT-PCR. The screening method is characterized by simultaneously performing two or three PCRs in a single tube using primers that are designed to correspond to poorly conserved regions selected by a homology search between nucleotide sequences of genes of an attenuated influenza virus and those of a virulent virus, thereby effectively screening a 6:2 reassortant virus containing six internal genes from an attenuated influenza virus and two external genes from a virulent virus.

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# SCREENING METHOD OF REASSORTANT INFLUENZA VIRUSES USING PRIMER DEPENDENT MULTIPLEX RT-PCR

## Technical Field

The present invention relates to a screening method of genes of reassortant influenza viruses in a reassortant influenza virus preparation process by using a difference between primers in binding specificity to templates in multiplex RT-PCR.

## Background Art

Influenza is an acute respiratory illness caused by infection of influenza viruses, which exhibits upper and lower respiratory symptoms as well as fever, headache, muscle aches and asthenia. Influenza infection occurs almost every winter with various symptoms and pathogenic states according to pandemic subtypes of influenza viruses. Since influenza infection is highly epidemic and often causes pneumonia and complications associated with heart and lung diseases with high mortality, the elderly, young children and even adults are recommended to be vaccinated with influenza vaccines.

Influenza viruses belong to the family *Orthomyxoviridae* and contain eight negative-sense RNA segments encoding PB2, PB1, PA, HA, NP, NA, M and NS. Among

the proteins, two outer envelope proteins, hemagglutinin (hereinafter, referred to as simply "HA") and neuraminidase (hereinafter, referred to as simply "NA") are potent immunogens that induce production of antibodies against influenza viruses, and are modified by the antigenic shift and drift processes. The high variability of HA and NA allows an influenza virus to escape the immunity formed against other influenza viruses within an identical subtype. Since immune responses induced by an influenza virus typically disappear in a short time, immunization against viruses predicted to spread should be newly performed every season.

In the case of influenza vaccines, inactivated vaccines against some influenza virus strains (two strains for type A and one strain for type B), epidemics of which have been expected by the World Health Organization (WHO), are typically used. Three inactivated influenza vaccines are widely used: whole virus vaccines using whole inactivated virions of influenza viruses; influenza split vaccines prepared by disrupting and inactivating influenza virus particles while maintaining their antigenicity; and influenza HA vaccines prepared by using only hemagglutinin isolated from influenza viruses. Viruses used in preparing these inactivated vaccines are typically cultured in fertilized eggs, and recently, their production by cell culturing has been attempted.

However, influenza viruses epidemics which have been

predicted by WHO have low proliferation capacity in fertilized eggs, resulting in low productivity. Productivity can be improved by reassorting a target strain with a virus strain adapted for high growth in fertilized eggs, thus generating a 6:2 reassortant virus containing genome segments encoding the viral surface antigens, HA and NA, which are derived from the target strain, and the remaining six internal genome segments from the high-yielding strain. This method has been applied to improve vaccine productivity in fertilized eggs for a long time (Influenza, Plenum Medical Book Company, 291, 1987).

Inactivated vaccines provide protective efficacy rates greater than 70% in young adults and are effective in reducing hospitalization due to respiratory illnesses and secondary infections such as pneumonia. However, they have disadvantages of having short immunity duration and being intramuscularly administered and not inducing production of mucosal secretory antibodies (sIgA) responsible for initial protection against viral infections. The disadvantages further include that the inactivated vaccines do not induce the CTL immunity sufficiently leading to death of infected cells.

Many efforts were made to develop live influenza vaccines that overcome the disadvantages of the inactivated vaccines, are highly immunogenic and are administered intranasally through the nasal mucosa. The first effort was made in the Soviet Union in 1965, resulting in the

development of a live vaccine virus strain using a cold-adapted attenuated virus strain, and, at present, still many studies associated with live influenza vaccines have been performed (Rev Roum Infra microbiol 2, 179-89, 1965). In the Soviet Union, a reassortant virus was developed by using an A/Leningrad/134/47/57(H2N2) virus as a donor virus strain for a live vaccine. In America, clinical trials for a live vaccine containing a reassortant virus strain that had been prepared by employing an A/Ann Arbor/6/60(H2N2) virus as a donor virus strain were completed, and the live vaccine was licensed for vaccination for people between the ages of 5 and 49.

To accomplish high productivity and safety of inactivated vaccines and live vaccines, a reassortant virus should be prepared by reassorting a target virus with a strain having high productivity in fertilized eggs or an attenuated virus strain. Since combinations occur randomly between eight RNA segments of influenza viruses during reassortant virus preparation, a maximum of $2^8$, that is, 256, different reassortant viruses can be produced. Thus, it is very important to establish a rapid and efficient selection method of 6:2 reassortant, that possesses the attenuation property of the donor virus and contains the outer proteins of virulent viruses, HA and NA virus, from the produced reassortant viruses.

A 6:2 reassortant virus is prepared as follows. First, a virulent virus is mixed with a donor virus in a

predetermined ratio, and the mixed viruses are inoculated in 11-day-old fertilized eggs. The eggs are incubated at a low temperature to produce reassortant viruses by random combinations. To select only viruses having the cold adaptation property of the donor virus and containing outer envelope proteins from the virulent virus, sub-culturing in fertilized eggs is performed at a low temperature in the presence of an antibody specific to the donor virus. A single virus clone is obtained by plaque isolation in the presence of an antibody specific to the donor virus, and is grown again in fertilized eggs to be propagated. HA and NA on the multiplied virus can be rapidly analyzed by a hemagglutinin inhibition assay (hereinafter, referred to simply as "HI") and a neuraminidase inhibition assay (hereinafter, referred to simply as "NI"). However, the remaining six internal genes are genetically analyzed.

For analysis of genes of reassortant influenza viruses, RNA genome can be directly analyzed by a method based on analyzing a difference of single-stranded RNA molecules in migration on a PAGE (polyacrylamide gel electrophoreses) gel, which is caused by different RNA sequences (J. Virol. 29,1142-1148,1979), Northern blotting and Crot analysis (RNA-DNA hybridization) (J. Gen. Virol. 64, 2611-2620, 1983; Vaccine 3, 267-273, 1985). However, these methods are problematic in terms of direct use of easily degradable RNA molecules. Also, they are cumbersome because RNA should be run on a PAGE gel for a long time.

Further, since the methods analyze a difference in nucleotide sequences of genes based on the difference in migration on a gel, they often lead to inaccurate results.

To solve these problems, a RFLP (Restriction Fragment Length Polymorphism) method was introduced, which analyses the gene by a combination of Reverse Transcription-Polymerase Chain Reaction (hereinafter, referred to simply as "RT-PCR") and the specificity of restriction enzymes (J. Virol. Methods 52, 41-49, 1995). RFLP is the most commonly used method for comparing patterns obtained by digesting genomes of donor and virulent viruses with restriction enzymes. However, there are drawbacks with the RFLP method, as follows. Eight RNA genome segments of influenza viruses should be individually isolated and amplified by RT-PCR and then digested with restriction enzymes. The nucleotide sequence of a virulent virus should first be analyzed to select proper restriction enzymes. Also, it takes many times to find proper restriction enzymes capable of distinguishing a donor virus and a virulent virus. Further, when variations occur in specific nucleotide sequences recognized by selected restriction enzymes, inaccurate results are obtained.

In addition to the above application, RT-PCR has been used for detecting influenza viruses and identifying subtypes thereof. However, when used for screen reassortant viruses, the RT-PCR method has a limitation in rapidly, effectively distinguishing genes from a donor virus and a virulent virus.

In this regard, the present inventors developed a method of rapidly distinguishing only a reassortant virus capable of being used as a live vaccine from various combinations of a virus by designing a plurality of primers specific to a donor virus and improving a PCR method, and completed the present invention by practically applying the method for screening reassortant viruses with two to four genes amplified in an identical reaction and confirming its effectiveness.

## Disclosure of the Invention

In an aspect, the present invention provides oligonucleotide primers allowing rapid screening of reassortants of a cold-adapted influenza virus, HTCA-A101 (Accession No.: KCTC 0400 BP), and a type A influenza virus.

In another aspect, the present invention provides a screening method of reassortant viruses by a multiplex RT-PCR method using pairs of the oligonucleotide primers.

## Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the results of RT-PCR performed using PB2 primer sets listed in Table 1 to amplify PB2 of eight RNA segments of an attenuated virus strain HTCA-A101 (M: molecular marker; lanes 1 to 9: PB2 primer sets 1 to 9 according to the present invention; and C: positive PB2 primer set);

Fig. 2 shows the results of RT-PCR performed using PB2 primer sets listed in Table 1 to amplify PB2 of eight RNA segments of each of four virulent viruses, A/New Caledonia/20/99 (H1N1), A/Moscow/10/99 (H3N2), A/Beijing/262/95 (H1N1) and A/Shangdong/9/93 (H3N2), wherein the numbers and capitals in an upper part are the same as those in Fig. 1;

Fig. 3 shows the results of multiplex PCR performed concurrently under identical conditions using as a template cDNA of each two virulent viruses, A/New Caledonia/20/99 (H1N1) and A/Moscow/10/99 (H3N2), wherein, the A of Fig. 3 shows the results for a virulent virus, A/New Caledonia/20/99 (H1N1) [M: molecular marker; lanes 1 to 3 shows the results for eight RNA segments of the attenuated virus strain, which are obtained using specific primer sets, lane 1: PB2, PB1 and NP; lane 2: M, NS and PA; and lane 3: HA and NA, in detail, lane 1: PB2-2 (1002 bp), NP-4 (853 bp) and PB1-6 (559 bp); lane 2: PA-5 (730 bp), NS-5 (521 bp) and M-6 (326 bp); and lane 3: HA-4 (1086 bp) and NA-4 (504 bp); lanes 4 to 6 show the results obtained using the same primer sets as in lanes 1 to 3; lanes 7 to 14 show the results of positive controls for

eight RNA segments, wherein lanes 13 and 14 show the results obtained using specific primer sets for H1 and N1 subtypes of A/New Caledonia/20/99 (H1N1)], and the B of Fig. 3 shows the results for another virulent virus, A/Moscow/10/99 (H3N2) [M: molecular marker; lanes 1 to 3 show the results for eight RNA segments of the attenuated virus strain, which are obtained using specific primer sets, lane 1: PB2, PB1 and NP; lane 2: M, NS and PA; and lane 3: HA and NA, in detail, lane 1: PB2-2 (1002 bp), NP-4 (853 bp) and PB1-6 (559 bp); lane 2: PA-5 (730 bp), NS-5 (521 bp) and M-6 (326 bp); and lane 3: HA-4 (1086 bp) and NA-4 (504 bp); lanes 4 to 6 show the results obtained using the same primer sets as in lanes 1 to 3; lanes 7 to 14 show the results of positive controls for eight RNA segments, wherein lanes 13 and 14 show the results obtained using specific primer sets for H3 and N2 subtypes of A/Moscow/10/99 (H3N2)];

Fig. 4 shows analysis results using a screening method according to the present invention for viruses obtained by infecting a single fertilized egg with a virulent virus A/New Caledonia/20/99 (H1N1) and an attenuated virus strain, growing the viruses in the egg, reacting obtained viruses with an antibody against the attenuated virus strain and isolating plaques, wherein four lanes show the results for a single virus (lanes 1 to 3: the results of multiplex PCR to investigate the origin of eight genes; lane 4: the results of PCR for M (649 bp) of eight genes as a positive control to monitor the presence or

absence of genes during RNA preparation and RT-PCR), which are obtained using primer sets, lane 1: PB2-2 (1002 bp), NP-4 (853 bp) and PB1-6 (559 bp); lane 2: PA-5 (730 bp), NS-5 (521 bp) and M-6 (326 bp); lane 3: HA-4 (1086 bp), and NA-4 (504 bp); and lane 4: Positive M (649 bp).

Best Mode for Carrying Out the Invention

To prepare a reassortant virus possessing the attenuation property of a donor virus and containing outer envelope proteins from a virulent virus, it is most important to select a virus strain including internal genes from the donor virus and outer envelope protein genes from the virulent virus by analyzing genes of obtained reassortant viruses to identify the origin of the genes.

However, primer design for determining whether genes are derived from the donor virus or the virulent virus requires a long time. Also, RNA isolation from a plurality of reassortant viruses obtained by various combinations and analysis of the isolated RNA require a lot of effort and time. With regard to the situation that live vaccine virus strains should be prepared using viruses recommended by WHO and produced for a short period of time (about one to two months) in the Northern Hemisphere, related studies should be focused on such work and to develop methods allowing vaccine production in a short period of time.

In this regard, the present inventors designed a

plurality of primers specific to an attenuated donor virus by analyzing the influenza virus database accumulated so far, and selected primers allowing a distinction between a donor virus and a virulent virus by RT-PCR for the two viruses in a matrix form. Introduction of a multiplex PCR method using the selected primers resulted in the development of a method capable of rapidly identifying a virus of interest by only three or four reactions.

Therefore, in an aspect, the present invention aims to provide primer sets specifically reacting with a donor virus HTCA-A101 and a PCR method using the primer sets for screening 6:2 reassortants of a donor virus and a type A influenza virus.

In another aspect, the present invention aims to provide a multiplex PCR method which is simpler and more economical than the case of simultaneously performing multiple PCRs.

In addition, the use of the multiplex PCR method according to the present invention may lead to rapid screening for 6:2 reassortant viruses.

In more detail, the present invention provides a PCR method for screening a 6:2 reassortant of a donor virus and a virulent virus, comprising the steps of designing PCR primer sets producing PCR products from a genome of an attenuated donor virus while not producing PCR products from a genome of a virulent virus on amplification for eight RNA genome segments of influenza viruses; infecting a

fertilized egg with the virulent virus and the attenuated donor virus and allowing random reassortment between the two viruses to produce reassortant viruses; preparing cDNA by reverse transcription of an RNA genome of each of the reassortant viruses; performing PCR for the eight RNA genome segments using the reassortant virus cDNA and a primer set for each of the eight RNA genome segments, wherein the primer set allows PCR products to have different lengths; and selecting a reassortant virus with which six internal RNA genome segments (PB2, PB1, PA, NP, M and NS) are amplified while two RNA genome segments encoding outer envelope proteins (HA and NA) are not amplified in a PCR using primer sets specific to the attenuated donor virus.

More preferably, the present invention provides a PCR method using an HTCA-A101 strain (KCTC 0400 BP) as an attenuated donor virus.

In addition, in the present invention, type A influenza viruses, A/Moscow/10/99, A/New Caledonia/20/99, A/Shangdong/9/93 and A/Shangdong/6/86, are used as target virulent viruses for screening for illustrative purpose, but the present invention is not limited to these viruses.

The present invention also provides a multiplex PCR method in which amplification for the eight genes is performed in three or four tubes while two or three PCRs occur simultaneously in a single tube.

In more detail, the present invention provides a

multiplex PCR method in which primer sets for PB2, PB1 and NP; M, NS and PA; and HA and NA are put into, respectively, a first tube, a second tube and a third tube, and, when PCRs are carried out for a reassortant virus in the tubes, PCR products are produced only in the first and second tubes. Herein, since HA and NA are major immunogenic proteins, when the reassortant virus is to be used as an inactivated or live vaccine, the two proteins should be derived from the virulent virus.

Therefore, preferably, the present invention provides a multiplex PCR method of screening a 6:2 reassortant of a donor virus and a virulent virus.

The PCR and RT-PCR according to the present invention may be carried out by a PCR method generally known in the art, preferably by one cycle of denaturation at 94°C for 5 min, and 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and elongation at 72°C for 1 min 20 sec, but the present invention is not limited to these PCR conditions.

The present invention also provides primer sets listed in Table 1 for use in the PCR method, which are specific for eight RNA genome segments including six internal genes and two outer envelope proteins of the attenuated donor virus.

An influenza virus as mentioned in the present invention contains six non-immunogenic genes, PB2, PB1, PA, NP, M and NS, and two immunogenic genes, HA and NA. The PCR

method according to the present invention allows rapid screening for a 6:2 reassortant virus for use as an inactivated or live vaccine by employing specific primer sets producing PCR products specific for an attenuated donor virus HTCA-A101 while not producing PCR products specific for a virulent virus.

Hereinafter, the present invention will be described in more detail.

1. Oligonucleotide primer design

A homology search was first performed between the donor virus and virulent viruses using influenza virus sequence database, and primers were designed in regions with low homology. Primers were designed to have a length of about 19-21 bases. At least 2 or 3 bases at a 3'-end of each of the primers corresponded to a region having different bases in a continuous sequence of the donor virus and the virulent virus. Also, the primers were designed to have an annealing temperature of about 58-60°C for optimal multiplex PCR performance. In target sequences, regions having variations were preferentially selected, and regions having a run of an identical base were excluded to provide donor virus-specific primers. The resulting primers that have a sequence corresponding to the regions of low homology were used for screening according to the present invention. Regions of high homology (100% homology based on the database) in the target sequences were used for the

design of positive control primers that were used in RT-PCR. In detail, in the present invention, a cold-adapted A/X-31 virus strain, HTCA-A101 (hereinafter, referred to simply as "A101") was used as a donor virus. Nucleotide sequences of type A influenza viruses for homology search with the donor virus were obtained from an influenza virus database at a web site ((http://www.flu.lanl.gov)), and a homology search was carried out using nucleotide sequences of H3N2 and H1N1 subtypes infecting humans. A homology search was carried out not by a 1:1 alignment between A101 and a virulent virus but by a multiple alignment between A101 and viruses registered in the database, particularly, all human-infecting viruses having a complete sequence registered in the database. Consistent criteria were difficult to apply for all target sequences on selection of specific regions for primer design because genome segments have different sequence homology levels. To easily detect PCR products according to their size, regions providing the largest difference in size of PCR products were selected in the target sequences.

2. RT-PCR analysis of templates from A101 and the virulent virus

First, optimal conditions for amplification of all eight RNA genome segments of A101 were established using the primer sets as prepared above. PCR products obtained from a genome from A101 were compared with those obtained

from a genome from the virulent virus under the same conditions.

3. Establishment of optimal conditions for multiplex RT-PCR

After primers producing PCR products from a genome of the donor virus strain while not producing PCR products from a genome of the virulent virus were selected, a set of the selected primers was selected for each of the eight genes to be amplified, and eight PCRs were designed to be carried out in three tubes.

Conventional techniques associated with multiplex RT-PCR conditions are concentrated on the reduction of interactions of primers with PCR products to produce multiple PCR products in a single template. In contrast, the present invention is characterized in that different PCRs for the eight genes happen simultaneously on eight templates from the virulent virus. In addition, to distinguish PCR products from each other on an agarose gel, combinations of primers were determined with regard to the size of PCR products. This multiplex RT-PCR allows the identification of the origin of genes of a reassortant virus, and its results were correlated with results of partial nucleotide sequence analysis.

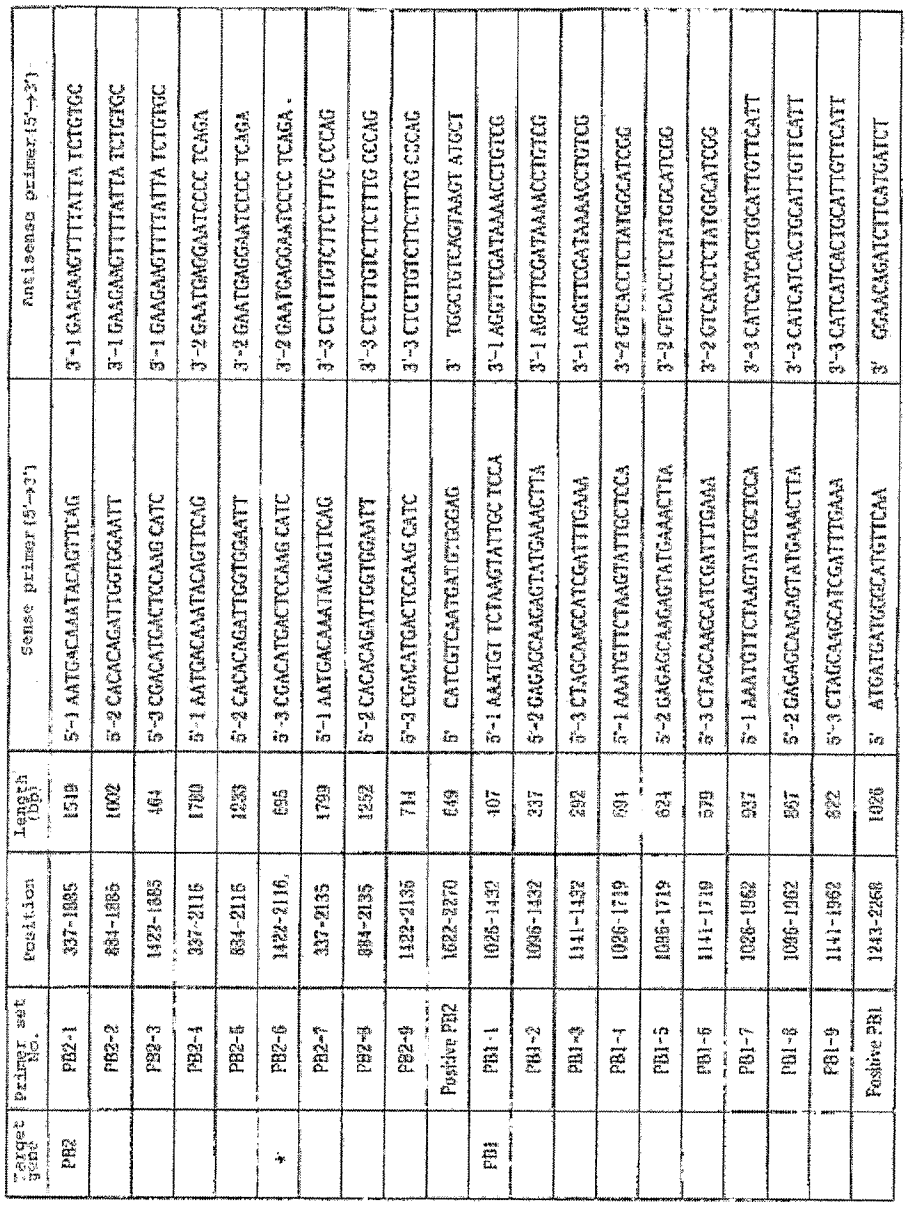
A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Design and synthesis of oligonucleotide primers

Influenza A viruses contain eight negative-sense RNA segments encoding PB2, PB1, PA, HA, NP, NA, M and NS. The eight genes were subjected to a homology search between human-infecting H3N2 and H1N1 subtypes and an attenuated virus, HTCA-A101, using nucleotide sequences obtained from an influenza virus database at a web site ((http://www.flu.lanl.gov)). Multiple alignment was carried out with a VECTOR NTI's aligment program. Six regions of low homology were selected for each gene: three for 5' primers (sense primers) and three for 3' primers (antisense primers). By various combinations of the primers, nine primer sets were designed for each of PB2, PB1, PA, NP, M and NS genes. For HA and NA genes, four regions were selected for each gene: two for 5' primers and two for 3' primers. Using these primer sets, PCR products specific for a template from the HTCA-A101 virus were produced. In contrast, positive control primers were designed to correspond to regions of high homology. One positive control primer was designed for each of PB2, PB1, PA, NP, M and NS, and two positive control primers were designed for each of HA and NA genes. Nucleotide sequences of the primers and size of PCR products are given in Tables 1a to 1d, below.

Nucleotide sequences of the primers and size of amplified products

TABLE 1a

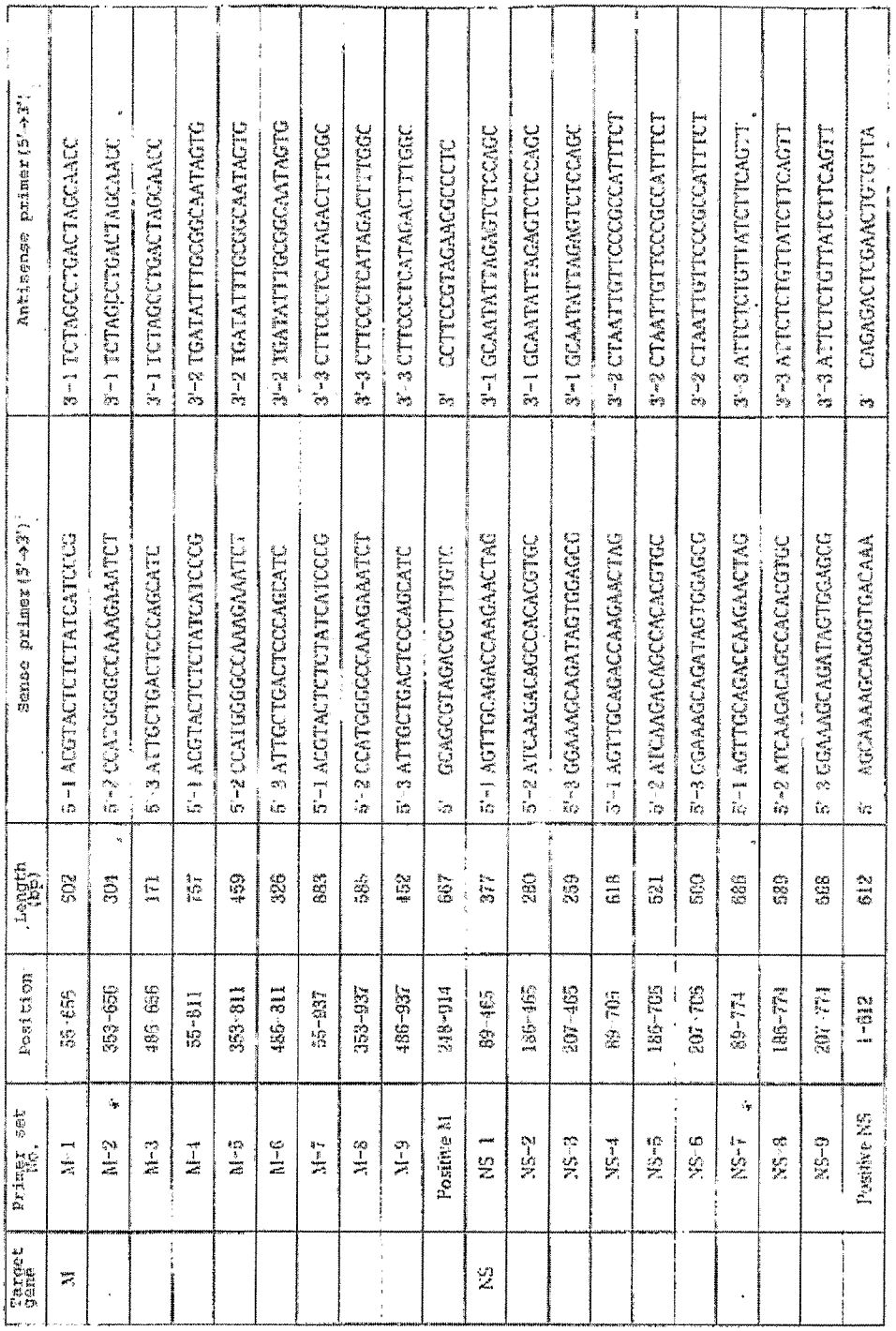


| Target gene | Primer set No. | Position | Length (bp) | Sense primer(5'→3') | Antisense primer(5'→3') |
|---|---|---|---|---|---|
| PB2 | PB2-1 | 337-1985 | 1519 | 5'-1 AATGACAAATACAGTTCAG | 3'-1 GAAGAAGTTTTTATTA TCTGTGC |
| | PB2-2 | 484-1985 | 1002 | 5'-2 CACACAGATTGGTGGAATT | 3'-1 GAAGAAGTTTTTATTA TCTGTGC |
| | PB2-3 | 1422-1885 | 464 | 5'-3 CGACATGACTCCAAG CATC | 3'-1 GAAGAAGTTTTTATTA TCTGTGC |
| | PB2-4 | 337-2116 | 1780 | 5'-1 AATGACAAATACAGTTCAG | 3'-2 GAATGAGGAATCCCC TCAGA |
| | PB2-5 | 884-2116 | 1233 | 5'-2 CACACAGATTGGTGGAATT | 3'-2 GAATGAGGAATCCCC TCAGA |
| | PB2-6 | 1422-2116 | 695 | 5'-3 CGACATGACTCCAAG CATC | 3'-2 GAATGAGGAATCCCC TCAGA |
| | PB2-7 | 337-2135 | 1799 | 5'-1 AATGACAAATACAGTTCAG | 3'-3 CTCTTGTCTTCTTTG CCCAG |
| | PB2-8 | 884-2135 | 1252 | 5'-2 CACACAGATTGGTGGAATT | 3'-3 CTCTTGTCTTCTTTG CCCAG |
| | PB2-9 | 1422-2135 | 714 | 5'-3 CGACATGACTCCAAG CATC | 3'-3 CTCTTGTCTTCTTTG CCCAG |
| | Positive PB2 | 1622-2270 | 649 | 5' CATCGTCAATGATGTGGGAG | 3' TGGCTGTCAGTAAGT ATGCT |
| PB1 | PB1-1 | 1026-1432 | 407 | 5'-1 AAATGT TCTAAGTATTGC TCCA | 3'-1 AGGTTCGATAAAACCTGTCG |
| | PB1-2 | 1026-1432 | 337 | 5'-2 GAGAGCAAGAGTATGAAACTTA | 3'-1 AGGTTCGATAAAACCTGTCG |
| | PB1-3 | 1141-1432 | 292 | 5'-3 CTAGCAAGCATCGATTTGAAA | 3'-1 AGGTTCGATAAAACCTGTCG |
| | PB1-4 | 1026-1719 | 691 | 5'-1 AAATGTTCTAAGTATTGCTCCA | 3'-2 GTCACCTCTATGGCATCGG |
| | PB1-5 | 1026-1719 | 624 | 5'-2 GAGAGCAAGAGTATGAAACTTA | 3'-2 GTCACCTCTATGGCATCGG |
| | PB1-6 | 1141-1719 | 579 | 5'-3 CTAGCAAGCATCGATTTGAAA | 3'-2 GTCACCTCTATGGCATCGG |
| | PB1-7 | 1026-1962 | 937 | 5'-1 AAATGTTCTAAGTATTGCTCCA | 3'-3 CATCATCACTGCATTGTTCATT |
| | PB1-8 | 1026-1962 | 937 | 5'-2 GAGAGCAAGAGTATGAAACTTA | 3'-3 CATCATCACTGCATTGTTCATT |
| | PB1-9 | 1141-1962 | 822 | 5'-3 CTAGCAAGCATCGATTTGAAA | 3'-3 CATCATCACTGCATTGTTCATT |
| | Positive PB1 | 1243-2268 | 1026 | 5' ATGATGTGGGCATGTTCAA | 3' GGAAGAGATCTTCATGATCT |

TABLE 1b

| Target gene | Primer set No. | Position | Length (bp) | Sense primer(5'→3') | Antisense primer(5'→3') |
|---|---|---|---|---|---|
| PA | PA-1 | 257-1336 | 1080 | 5'-1 TAATCGAGGGAAGAGATCGC | 3'-1 TGTGTTCAATTGGAGCCACA |
| | PA-2 | 811-1336 | 526 | 5'-2 ACAACACCACGACCACTTAG | 3'-1 TGTGTTCAATTGGAGCCACA |
| | PA-3 | 971-1336 | 366 | 5'-3 GATGGAAGGAACCCAATGTT | 3'-1 TGTGTTCAATTGGAGCCACA |
| | PA-4 | 257-1541 | 1285 | 5'-1 TAATCGAGGGAAGAGATCGC | 3'-2 TTTATGATGAAACCAACAAGC |
| | PA-5 | 811-1541 | 731 | 5'-2 ACAACACCACGACCACTTAG | 3'-2 TTTATGATGAAACCAACAAGC |
| | PA-6 | 971-1541 | 571 | 5'-3 GATGGAAGGAACCCAATGTT | 3'-2 TTTATGATGAAACCAACAAGC |
| | PA-7 | 257-1710 | 1454 | 5'-1 TAATCGAGGGAAGAGATCGC | 3'-3 GAACATGGGCCTTGAAACCT |
| | PA-8 | 811-1710 | 900 | 5'-2 ACAACACCACGACCACTTAG | 3'-3 GAACATGGGCCTTGAAACCT |
| | PA-9 | 971-1710 | 740 | 5'-3 GATGGAAGGAACCCAATGTT | 3'-3 GAACATGGGCCTTGAAACCT |
| | Positive PA | 34-366 | 333 | 5' GTAGTCTGCCTTTGTGGCC | 3' TCTTGTCTTATGGTCAATAG |
| NP | NP-1 | 327-1120 | 794 | 5'-1 ACCTATATACAGGAGAGTAAAC | 3'-1 AAAGCTTCCCTCTTGGGAGC |
| | NP-2 | 605-1120 | 516 | 5'-2 GAACAATGGTGATGGAATTGG | 3'-1 AAAGCTTCCCTCTTGGGAGC |
| | NP-3 | 905-1120 | 216 | 5'-3 GTGGGTACGACTTTGAAAGG | 3'-1 AAAGCTTCCCTCTTGGGAGC |
| | NP-4 | 327-1181 | 855 | 5'-1 ACCTATATACAGGAGAGTAAAC | 3'-2 AGTGTACTTGATTCCATAGTC |
| | NP-5 | 605-1181 | 577 | 5'-2 GAACAATGGTGATGGAATTGG | 3'-2 AGTGTACTTGATTCCATAGTC |
| | NP-6 | 905-1181 | 277 | 5'-3 GTGGGTACGACTTTGAAAGG | 3'-2 AGTGTACTTGATTCCATAGTC |
| | NP-7 | 327-1329 | 1003 | 5'-1 ACCTATATACAGGAGAGTAAAC | 3'-3 TGCTGCCATAATGGTGGTTC |
| | NP-8 | 605-1329 | 725 | 5'-2 GAACAATGGTGATGGAATTGG | 3'-3 TGCTGCCATAATGGTGGTTC |
| | NP-9 | 905-1329 | 425 | 5'-3 GTGGGTACGACTTTGAAAGG | 3'-3 TGCTGCCATAATGGTGGTTC |
| | Positive NP | 392-1505 | 1114 | 5' TAAGGCGAATCTGCCGCAA | 3' TAAGATCCTTCATTACTGAT |

TABLE 1c



| Target gene | Primer set No. | Position | Length (bp) | Sense primer(5'→3') | Antisense primer(5'→3') |
|---|---|---|---|---|---|
| M | M-1 | 35-656 | 902 | 5'-1 ACGTACTCTCTATCATCCG | 3'-1 TCTAGCCTGACTAGCAACC |
| | M-2 | 353-656 | 304 | 5'-2 CCATGGGGCCAAAGAAATCT | 3'-1 TCTAGCCTGACTAGCAACC |
| | M-3 | 486-656 | 171 | 5'-3 ATTGCTGACTCCCAGCATC | 3'-1 TCTAGCCTGACTAGCAACC |
| | M-4 | 35-811 | 757 | 5'-1 ACGTACTCTCTATCATCCCG | 3'-2 TGATATTTGCGGCAATAGTG |
| | M-5 | 353-811 | 459 | 5'-2 CCATGGGGCCAAAGAAATCT | 3'-2 TGATATTTGCGGCAATAGTG |
| | M-6 | 486-811 | 326 | 5'-3 ATTGCTGACTCCCAGCATC | 3'-2 TGATATTTGCGGCAATAGTG |
| | M-7 | 35-937 | 883 | 5'-1 ACGTACTCTCTATCATCCCG | 3'-3 CTTCCTCATAGACTTTGGC |
| | M-8 | 353-937 | 585 | 5'-2 CCATGGGGCCAAAGAAATCT | 3'-3 CTTCCTCATAGACTTTGGC |
| | M-9 | 486-937 | 452 | 5'-3 ATTGCTGACTCCCAGCATC | 3'-3 CTTCCTCATAGACTTTGGC |
| | Positive M | 348-914 | 567 | 5' GCAGCGTAGACGCTTTGTC | 3' CCTTCCGTAGAAGCCCTC |
| NS | NS 1 | 89-465 | 377 | 5'-1 AGTTGCAGACCAAGAACTAG | 3'-1 GCAATATTAGAGTCTCCAGC |
| | NS-2 | 186-465 | 280 | 5'-2 ATCAAGACAGCCACACGTGC | 3'-1 GCAATATTAGAGTCTCCAGC |
| | NS-3 | 207-465 | 259 | 5'-3 GGAAAGCAGATAGTGGAGCG | 3'-1 GCAATATTAGAGTCTCCAGC |
| | NS-4 | 89-705 | 618 | 5'-1 AGTTGCAGACCAAGAACTAG | 3'-2 CTAATTGTTCCCGCCATTTCT |
| | NS-5 | 186-705 | 521 | 5'-2 ATCAAGACAGCCACACGTGC | 3'-2 CTAATTGTTCCCGCCATTTCT |
| | NS-6 | 207-705 | 500 | 5'-3 GGAAAGCAGATAGTGGAGCG | 3'-2 CTAATTGTTCCCGCCATTTCT |
| | NS-7 | 89-774 | 686 | 5'-1 AGTTGCAGACCAAGAACTAG | 3'-3 ATTCTCTGTTATCTTCAGTT |
| | NS-8 | 186-774 | 589 | 5'-2 ATCAAGACAGCCACACGTGC | 3'-3 ATTCTCTGTTATCTTCAGTT |
| | NS-9 | 207-774 | 568 | 5'-3 GGAAAGCAGATAGTGGAGCG | 3'-3 ATTCTCTGTTATCTTCAGTT |
| | Positive NS | 1-612 | 612 | 5' AGCAAAAGCAGGGTGACAAA | 3' CAGAGACTCGAACTGTGTTA |

TABLE 1d

| Target gene | Primer set No. | Position | Length (bp) | Sense primer(5'→3') | Antisense primer(5'→3') |
|---|---|---|---|---|---|
| HA | HA-1 | 447-1277 | 831 | 5'-1 GGTTTCACTTGGACTGGGT | 3'-1 TGAGAATTCCTTTTGGATTTGA |
| | HA-2 | 447-1623 | 1177 | 5'-2 TGACCAAATCAGGAAGCA | 3'-1 TGAGAATTCCTTTTGGATTTGA |
| | HA-3 | 538-1277 | 740 | 5'-1 GGTTTCACTTGGACTGGGT | 3'-2 GCATCCAGTCTTTGTATCCA |
| | HA-4 | 538-1623 | 1086 | 5'-2 TGACCAAATCAGGAAGCA | 3'-2 GCATCCAGTCTTTGTATCCA |
| | Positive H1 Control | 33 629 | 597 | H1-5' ATGAAAGCAAAACTACTAGTTCA | H1-3' AGACGGGTGATGAACACCCCAT |
| | Positive H3 Control | 214 839 | 626 | H3 -5' CCTCAACAGGTAGAATATGCGC | H3-3' AGGAGCAATTAGATTCCCTGTG |
| NA | NA-1 | 227-1002 | 776 | 5'-1 AGATATGCCCCAAATTAGTG | 3'-1 CGATTGTTAGCCAGCCCATGCCA |
| | NA-2 | 227-1171 | 945 | 5'-2 TGCGTTTGTATCAATGGGAC | 3'-1 CGATTGTTAGCCAGCCCATGCCA |
| | NA-3 | 668-1002 | 335 | 5'-1 AGATATGCCCCAAATTAGTG | 3'-2 CTGCGATTTGGAATTAGGTG |
| | NA-4 | 668 1171 | 504 | 5'-2 TGCGTTTGTATCAATGGGAC | 3'-2 CTGCGATTTGGAATTAGGTG |
| | Positive N1 Control | 1-600 | 600 | N1-5' AGCAAAAGCAGGAGTTTAAAA | N1-3' CGATTGTTAGCCAGCCCATGCCA |
| | Positive N2 Control | 20 402 | 383 | N2-5' ATGAATTCCAAATCAAAAGATAA | N2-3' TTGCCAGGATGGCATGACACAT |
| Uni 12 | 1-AGCAAAGCAGG- 12 | | | | |

EXAMPLE 2: RNA genome isolation from influenza viruses

RNA was isolated using a TRIZOL LS reagent (GibcoBRL Company) directly from viral culture fluids. 900 μl of the TRISOL LS reagent was added to 300 μl of a viral culture fluid, and 200 μl of chloroform was added thereto. After being vigorously mixed for 10 sec, the fluid was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new tube and supplemented with an equal volume of isopropanol. After being allowed to stand at room temperature for 10 min, the tube was centrifuged at 12,000 rpm for 5 min. After the supernatant was discarded, 1 ml of 75% ethanol was added to the tube. After vortexing, the tube was centrifuged at 12,000 rpm for 10 min to precipitate RNA. The resulting RNA pellet was dissolved in RNase-free water, aliquotted and stored at -70°C until use.

In addition to the donor virus strain, influenza viruses used in this RNA isolation included four influenza A viruses recently causing epidemics, A/New Caledonia/20/99 (H1N1), A/Moscow/10/99 (H3N2), A/Singapore/6/86 (H1N1) and A/Shangdong/9/93 (H3N2).

EXAMPLE 3: Preparation of cDNA from influenza RNA genome viruses

After concentration of the isolated RNA was

determined by measuring absorbance at 260 nm, reverse transcription was carried out using a total of 2 μg of RNA as a template and a QIAGEN's Omniscript RT kit. A reaction mixture was prepared by mixing 12 μl of a RNA solution, 2 μl of 5 mM dNTP, 2 μl of 10× RT buffer, 2 μl of 10 pmol/ul primer (a conserved sequence in 3′ end of all eight negative-sense RNA segments of influenza viruses: 5′-AGCGAAAGCAGG-3′) and 1 μl of reverse transcriptase. The reaction mixture was incubated at 37° for 60 min to induce cDNA synthesis, and then was incubated at 94° for 5 min to inactivate reverse transcriptase. The synthesized cDNA was used in all PCRs for the eight genes.

EXAMPLE 4: Detection of PCR products for the donor virus and the virulent virus

PCR was carried out to determine whether PCR products are produced from templates from the virulent virus and the donor virus by the specificity of the primers. PCR was carried out using 1 μl of the cDNA solution prepared in Example 3 and a primer set for each gene prepared in Example 1. PCR conditions included denaturation at 94°C for 5 min, and 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and elongation at 72°C for 1 min 20 sec.

The amplified PCR products were separated on an agarose gel. As a result, in the case of the donor virus,

PCR products were produced from all combinations of primers corresponding to regions of low homology, that is, nine primer sets for each of the six internal genes and four primer sets for each of the two external genes encoding outer envelope proteins HA and NA. In contrast, in the case of the virulent viruses, although cDNA was found to be present by positive control reactions, PCR products were produced in fewer combinations. The results are given in Table 2, below. Among them, results for PB2 are shown in Fig. 2. These results indicate that the difference in PCR results between the donor virus and the virulent viruses on RT-PCR performance under the same conditions results from the specific binding of primers to a template from the donor virus.

TABLE 2

Results of RT-PCR using the primers and templates from the donor virus and the virulent viruses

| | PB2 | PB1 | PA | NP | M | NS | HA | NA |
|---|---|---|---|---|---|---|---|---|
| A/Moscow/10/99 (H3N2) | | | | | 2,3 | | | |
| A/Shangdong/9/93 (H3N2) | | | | | | | | |
| A/New Caledonia/20/99 (H1N1) | 2 | 1,2,4, 5,7,8 | 2,3,8, 9 | 1 | 1,2,3, 7,8,9 | 1,4,7 | | |
| A/Singapore/6/86 (H1N1) | 2,3,5, 6,9 | 1,2,3, 4,5,7, 8,9 | 1,2,3, 7,8,9 | 1,2,3, 5,7,8, 9 | 1,2,3, 4,5,6 | 1,3,4, 5,6,7 | | |

EXAMPLE 5: Multiplex RT-PCR detection

Multiplex RT-PCR was carried out using primer sets that were found in Example 4 to produce PCR products from a template from the donor virus while not producing PCR products from templates from the virulent viruses. The selected primer sets were divided into three groups: PB2-2 (1002 bp), NP-4 (853 bp) and PB1-6 (559 bp); PA-5 (730 bp), NS-5 (521 bp) and M-6 (326 bp); and HA-4 (1086 bp) and NA-4 (504 bp), and each group was put into a different tube. In the case of the virulent viruses, multiplex RT-PCR was carried out under the same PCR conditions as in the Monoplex PCR conditions except that primers were used in an amount as high as two to three times that in Monoplex PCR due to the segmented nature of influenza virus genome. As a result, in case of the donor virus, highly reproducible PCR products for all of the eight genes were produced by the amplification in the three tubes. In case of the influenza A/New Caledonia/20/99 (H1N1) and A/Moscow/10/99 (H3N2) viruses, PCR products from the positive control primers for eight genes were produced, but gene amplification did not happen in multiplex PCR tubes (see, Fig. 3). In contrast, multiplex PCR products for all of the eight genes were detected on amplification from the template from the donor virus strain (Fig. 3).

EXAMPLE 6: Genetic analysis of reassortant viruses

After reproducible multiplex PCR results from

templates from the donor and virulent viruses were obtained, the multiplex PCR method according to the present invention was practically applied for identification of reassortant viruses.

A virulent virus, A/New Caledonia/20/99(H1N1), and the donor virus simultaneously infected a fertilized egg and grown therein. As a result, various reassortant viruses were produced by random combination between RNA segments of the two viruses. From them, viruses containing HA and NA genes from the virulent virus and having the cold adaptation property of the donor virus were selected by culturing at a low temperature and by using an antibody specific to the donor virus. Since virulent viruses also have the growth property at a low temperature, genetic analysis is required to obtain a desired reassortant virus containing the internal genes from the donor virus.

Genetic analysis for the reassortant viruses were preformed according to the same method as in Example 5. The results are given in Table 3, below, and Fig. 4. A reassortant virus No. 7 was expected to be a 6:2 reassortant virus (in which PB2, PB1, PA, NP, M and NS genes are derived from the donor virus, and HA and NA genes are derived from the virulent virus), which produces PCR products for six internal genes while not producing PCR products for two external genes encoding outer envelope proteins. Another reassortant virus No. 2 was expected to be a 5:3 reassortant virus (in which PB2, PB1, NP, M and NS

genes are derived from the donor virus, and PA, HA and NA genes are derived from the virulent virus). The produced reassortant viruses include 4:4 reassortants (containing PA, PB1, NP and M genes from the donor virus and PB2, NS, HA and NA genes from the virulent virus) and a 7:1 reassortant (containing PB2, PB1, PA, NP, M, NS and NA genes from the donor virus and an HA gene from the virulent virus).

In addition, the 6:2 reassortant, reassortant virus No. 7, was subjected to nucleotide sequencing and PCR using nine primer sets for each gene. The results were compared with the results for the donor and virulent viruses (see, Table 4), and, as a result, they were correlated with the results obtained by amplification in four tubes (see, Fig. 4). These results indicate that the origin of eight genes of one recombinant virus can be identified by PCR in three or four tubes, thereby facilitating rapid identification for numerous reassortant viruses.

TABLE 3

Results of analysis according to the present method for viruses produced by reassortment between the virulent virus A/New Caledonia/20/99(H1N1) and the donor virus HTCA-A101

| Reassortant virus No. | No. of segments from donor virus : No. of segments from virulent virus (genes from donor virus) |
|---|---|
| 1 | 5:3 (PB2, PB1, NP, M, NS) |
| 2 | 5:3 (PB2, PB1, NP, M, NS) |
| 3 | 4:4 (PB1, NP, M, NS) |
| 4 | 4:4 (PB1, NP, PA, M) |
| 5 | 4:4 (PB1, NP, M, NS) |
| 6 | 4:4 (PB1, NP, M, NS) |
| 7 | 6:2 (PB2, PB1, PA, NP, M, NS) |
| 8 | 5:3 (PB2, PB1, NP, PA, NS) |
| 9 | 7:1 (PB2, PB1, PA, NP, M, NS, NA) |

TABLE 4

Comparison of nucleotide sequences obtained by partial sequencing of PCR products of the reassortant virus 7 with the known sequence of the donor virus HTCA-A101

| PCR products | Positions of the partial sequences | Size of the partial sequences | Homology (%) |
|---|---|---|---|
| PB2 (1622-2270) 649 bp | 1622-2270 | 649 bp | 100 |
| PB1 (1243-2268) 1026 bp | 1345-1906 2002-253 | 754 bp (562 + 192) | 100 |
| PA (34-507) 474 bp | 66-501 | 436 bp | 100 |
| NP (392-1505) 1114 bp | 60-469 | 410 bp | 99 |
| M (248-914) 649 bp | 444-849 | 406 bp | 100 |
| NS (1-612) 612 bp | 25-578 | 554 bp | 99 |
| HA (1-600) 600 bp | - | - | No significant homology |
| NA (1-600) 600 bp | - | - | No significant homology |

## Industrial Applicability

As described hereinbefore, the screening method of reassortant influenza viruses, which is based on performing multiplex RT-PCR using donor virus-specific primer sets designed according to the present invention, allows

selecting viruses having a desired combination from viruses formed by various combinations with small manpower for a short period of time at an intermediate stage of a preparation process of desired reassortant viruses. In addition, the screening method allows preparing desired reassortant viruses in a short time using a circulating virulent virus, thereby securing sufficient production and supply of live vaccine viruses.

## Claims

1. A screening method of influenza reassortant viruses by PCR, which comprises designing PCR primer sets producing PCR products for only internal genes of an attenuated donor virus on amplification for eight RNA genome segments of influenza viruses.

2. The screening method according to claim 1, wherein the screening method comprises:

designing PCR primer sets producing PCR products from a genome of an attenuated donor virus while not producing PCR products from a genome of a virulent virus on amplification for eight RNA genome segments of influenza viruses;

infecting a fertilized egg with the virulent virus and the attenuated donor virus and allowing random reassortment between the two viruses to produce reassortant viruses;

preparing cDNA by reverse transcription of an RNA genome of each of the reassortant viruses;

performing PCR for the eight RNA genome segments using the reassortant virus cDNA and a primer set for each of the eight RNA genome segments, wherein the primer set allows PCR products to have different lengths; and

selecting 6:2 reassortant virus with which six internal RNA genome segments (PB2, PB1, PA, NP, M and NS)

from the attenuated influenza virus are amplified while two RNA genome segments encoding outer envelope proteins (HA and NA) from the virulent virus are not amplified in a PCR using primer sets specific to the attenuated donor virus.

3. The screening method according to claim 1 or 2, wherein the attenuated donor virus is an HTCA-A101 strain (KCTC 0400 BP).

4. The screening method according to claim 1 or 2, using for screening influenza reassortant viruses a multiplex PCR method in which amplification of the eight genes is performed in three or four tubes while two or three PCRs occur simultaneously in a single tube.

5. Primer sets specifically binding to an attenuated donor virus, HTCA-A101, which are shown in Table 1.

**Fig. 1**

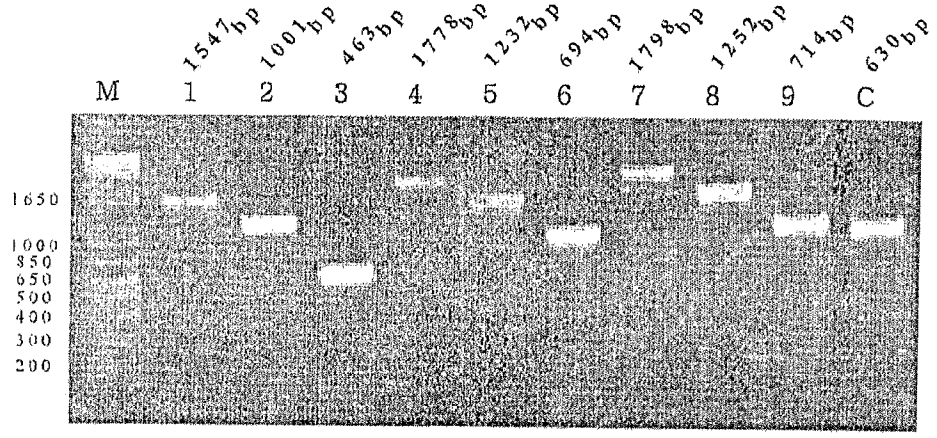

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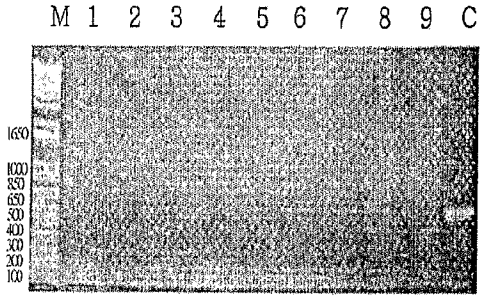
Fig. 2

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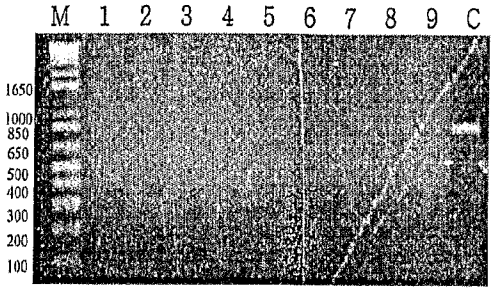


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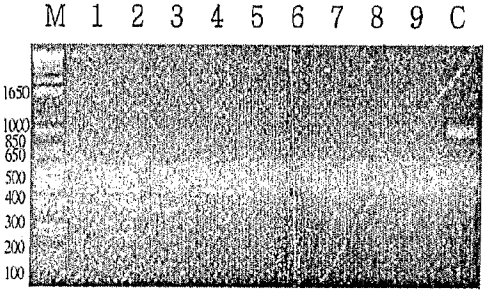


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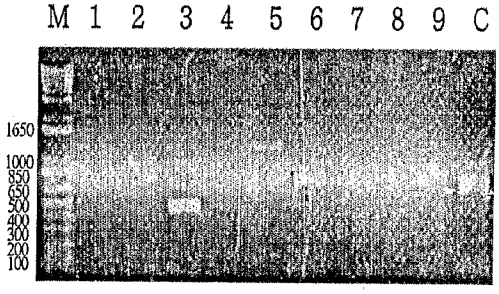
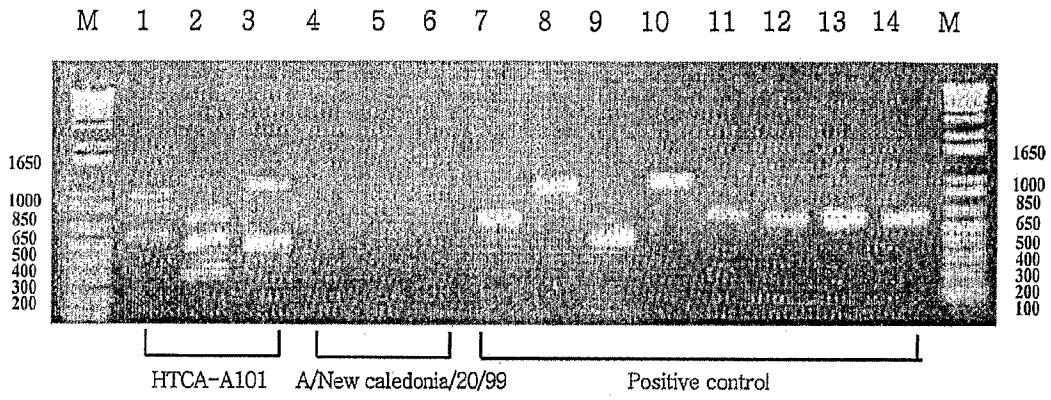



A/Singapore/6/86 (H1N1)

3/4

**Fig. 3**

A. A/New caledonia/20/99

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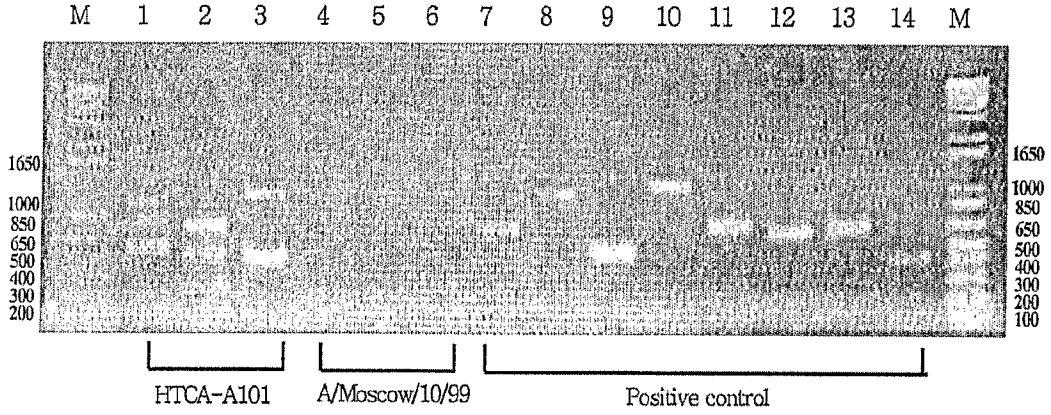
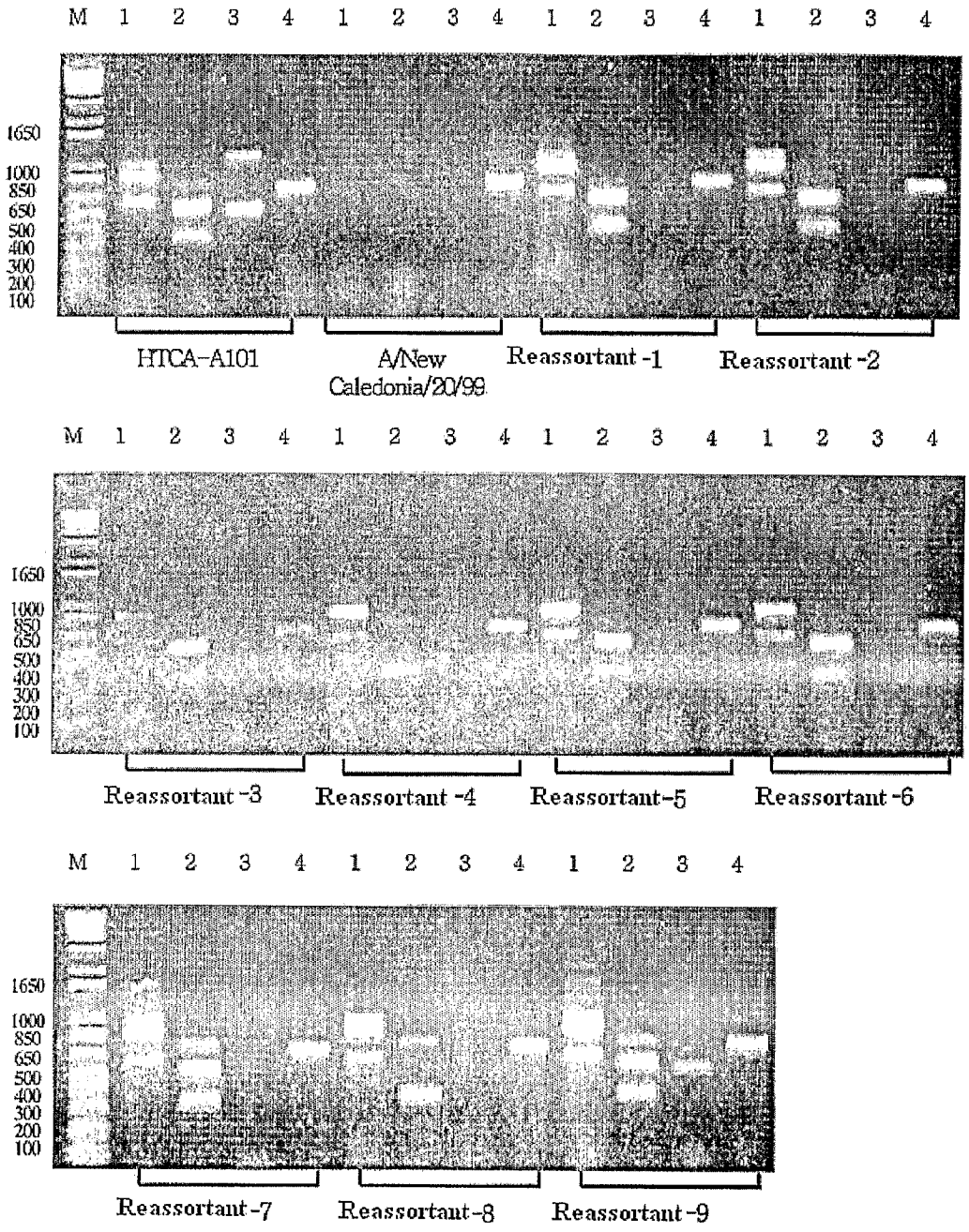


**Fig. 4**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Protheon

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<220>

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<210> 91
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<210> 96
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<400> 107
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<210> 118
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<210> 119
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<400> 120
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<400> 121
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<210> 128
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<210> 129
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<400> 131
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<400> 132
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rnational application No.
PCT/KR2004/001903

A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 C12Q 1/68**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 : C12Q 1/68, A61K 39/145

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and application for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, NPS, PAJ, DELPHION, WPINDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | J. of Clinical Microbiology, vol. 36, no. 10, pp. 2990-2995 (1998) see entire document | 1, 2, 4 |
| Y | J. of Clinical Microbiology, vol. 38, no. 2, pp. 839-845 (2000) see entire document | 1, 2, 4 |
| A | Analytical Biochemistry vol. 252, no. 1, pp. 24-32 (1997) see entire document | 1-5 |
| A | J. of Clinical Microbiology, vol. 39, no. 11, pp. 4097-4102 (2001) see entire document | 1-5 |
| A | Virus Research, vol. 32, no. 3, pp. 391-399 (1994) see entire document | 1-5 |

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 DECEMBER 2004 (15.12.2004) | **15 DECEMBER 2004 (15.12.2004)** |
| Name and mailing address of the ISA/KR<br>Korean Intellectual Property Office<br>920 Dunsan-dong, Seo-gu, Daejeon 302-701,<br>Republic of Korea<br>Facsimile No. 82-42-472-7140 | Authorized officer<br>BAIK, Kyong UP<br>Telephone No. 82-42-481-5596 |

Form PCT/ISA/210 (second sheet) (January 2004)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2004/001903

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] filed together with the international application in computer readable form

   [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: